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Transcriptional Portrait of Actinobacillus pleuropneumoniae during Acute Disease - Potential Strategies for Survival and Persistence in the Host

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Abstract

Background: Gene expression profiles of bacteria in their natural hosts can provide novel insight into the host-pathogen interactions and molecular determinants of bacterial infections. In the present study, the transcriptional profile of the porcine lung pathogen Actinobacillus pleuropneumoniae was monitored during the acute phase of infection in its natural host.

Methodology/Principal Findings: Bacterial expression profiles of A. pleuropneumoniae isolated from lung lesions of 25 infected pigs were compared in samples taken 6, 12, 24 and 48 hours post experimental challenge. Within 6 hours, focal, fibrino hemorrhagic lesions could be observed in the pig lungs, indicating that A. pleuropneumoniae had managed to establish itself successfully in the host. We identified 237 differentially regulated genes likely to encode functions required by the bacteria for colonization and survival in the host. This group was dominated by genes involved in various aspects of energy metabolism, especially anaerobic respiration and carbohydrate metabolism. Remodeling of the bacterial envelope and modifications of posttranslational processing of proteins also appeared to be of importance during early infection. The results suggested that A. pleuropneumoniae is using various strategies to increase its fitness, such as applying Na⁺ pumps as an alternative way of gaining energy. Furthermore, the transcriptional data provided potential clues as to how A. pleuropneumoniae is able to circumvent host immune factors and survive within the hostile environment of host macrophages. This persistence within macrophages may be related to urease activity, mobilization of various stress responses and active evasion of the host defenses by cell surface sialylation.

Conclusions/Significance: The data presented here highlight the importance of metabolic adjustments to host conditions as virulence factors of infecting microorganisms and help to provide insight into the mechanisms behind the efficient colonization and persistence of A. pleuropneumoniae during acute disease.

Introduction

Due to technical limitations and ethical considerations, most transcriptional studies of pathogenic bacteria have, until recently, been in vitro experiments intended to simulate microenvironments of the host in a simplified system. These studies have provided valuable insight into bacterial pathogenesis, but must be interpreted with caution, as the results of in vitro models are influenced by the model system used [1–3]. Real-life pathogenesis is a multifactorial process where the microbe is challenged by host immune factors and constant changes in nutrient availability [4]. The molecular mechanisms involved in bacterial pathogenicity can only be studied in depth in the natural host, where the gene expression profile accurately depicts the many concurrent responses that reflect the physiochemical conditions of the in vivo site of infection [5,6]. Due to technical improvements, it has now become possible to obtain prokaryotic mRNA from in vivo infections of a quality and quantity sufficient to perform whole genome transcriptional analysis [7–11].

The objective of this study was to gain a detailed understanding of the molecular basis of pathogenicity in the disease porcine pneumonia, by measuring the host-adapted genomic transcriptional response of A. pleuropneumoniae in its natural host during acute infection. This highly infectious respiratory disease is the cause of impaired animal welfare and serious economic losses in swine herds world-wide [12]. The etiological factor, A. pleuropneumoniae, is a Gram-negative, facultative anaerobic coccobacillus of the Pasteurellaceae family [13]. Macroscopically, the affected lung is characterized by fibrinohemorrhagic necrotizing bronchopneumonia and fibrinous pleuritis [14]. The infection can range from peracute disease with rapid death to chronic infection resulting in asymptomatic carriers [12]. Based on antigenic properties of the capsular polysaccharides and the cell wall lipopolysaccharides A. pleuropneumoniae has been divided into 15 serotypes, among which
some variance in virulence has been observed [14]. There are
three basic stages in the pathogenesis of porcine pneumonia:
colonization, subversion of host defense, and damage to host tissue
[12]. Some of the virulence factors involved in these stages have
been identified, such as adhesins, iron-acquisition factors, capsule
and lipopolysaccharides and in particular the RTX toxins, which
are major virulence factors of Pasteurella spp. [12,14]. Still,
important aspects of fundamental molecular processes in the
host-pathogen interactions of this disease remain to be elucidated,
e.g. which factors enable the successful survival and persistence of
A. pleuropneumoniae in the host. Many of the presently known
virulence factors have been identified by in vivo methods such as
signature tagged mutagenesis (STM), in vivo expression technology
(IVET) and selective capture of transcribed sequences (SCOTS)
[15–19].

The sequencing of the whole genome of a selection of serotypes of
A. pleuropneumoniae and the construction of genome-wide
microarrays has led to a number of interesting studies on the
traits underlying infection, for example during biofilm formation
and in environments mimicking the conditions in the lung during
early infection [20–22]. One of the few presently published
genome-wide transcriptional profiling studies of a bacterial
pathogen in its natural host was performed on A. pleuropneumoniae
[11]. Hitherto, both in vivo and in vitro studies of bacterial genomic
expression have included only a few samples. Here we present
what is, to the best of our knowledge, the first large scale
time-course in vivo transcriptome study of a bacterium in its natural host.
We compared expression profiles of A. pleuropneumoniae recovered
from the lungs of 25 pigs at four time-points during the first
48 hours after experimental challenge. In this study we gained
important information of the bacterial strategy during establish-
ment and survival in the host and identified putative new virulence
factors.

Results

In vivo transcriptome approach

We used a custom designed A. pleuropneumoniae NimbleGen
microarray to characterize the transcriptional profile of A.
pleuropneumoniae serotype 2 and serotype 6 in a time study 6, 12,
24 and 48 hours post infection (p.i.). Serotypes 2 and 6 represent
more than 90% of the clinical isolates originating from Denmark.
Visual macroscopic infection was confirmed established in 28 of
the 48 experimentally infected pigs. Details of the sampled
material are listed in Table S1. Cultivation from infected lungs
revealed that, except for 3 animals, co-infection mainly by
Pasteurella multocida but also Streptococcus suis and non-hemolytic
Escherichia coli, could be observed (Table S1). Most likely, these
bacteria were present before the inoculation with A. pleuropneumoniae.
As the array was designed to be highly specific for A.
pleuropneumoniae, comprising many short oligonucleotides for each
gene (covered by an average of 26.7 probes of a mean size of
48 bp), cross-hybridization of other bacteria than A. pleuropneumoniae
to the microarrays was expected to be minimal. This
assumption was supported by a Pearson’s correlation coefficient
of 0.93 between the pure cultures of A. pleuropneumoniae and the
mixed bacterial cultures; calculated from the median expression
values of each gene within the pure culture arrays versus the mixed
culture arrays.

Total RNA was extracted from three lung samples of each
infected animal (n = 84). The mRNA was linearly amplified to
obtain sufficient material for microarray analysis. To test whether
the data had been skewed by the amplification procedure, the
expression of 11 bacterial genes before and after mRNA
amplification was validated by quantitative real-time RT-PCR
(qPCR). Samples included three individual RNA extractions from
injected lung tissue of pig no. 33 and 55, before and after linear
amplification, respectively (n = 6). The results of the qPCR analysis
showed good correlation between expression of the selected genes
before and after amplification (Spearman’s rho 0.74, P<0.009 for
both animals) (Table S2).

Microarrays from three animals (triplicates; n = 9) were
discarded due to lack of sufficient signal detection, leaving 75
microarrays from 25 pigs for further downstream analysis. A
density plot of the 75 normalized microarrays is depicted in Figure
S1 and reveals a clear distinction between the background and the
expression signal. The reliability of the microarray data was
assessed by qPCR analysis. We selected a subset of 20 genes and
compared the results of the qPCR on cDNA from bacteria isolated
6 h (pigs no. 33, 36, 55 and 59) and 48 h (pigs no. 51, 54, 75 and
76) p.i., respectively. Of the 8 pigs, three biological samples
(independent mRNA extraction and cDNA synthesis) were
included in the analysis (n = 24). The qPCR results and microarray
data exhibited a high correlation coefficient (R² = 0.73) (Figure 1).

Genes differentially expressed during acute infection were
identified by comparing the whole genome transcriptional profiles of
bacteria recovered from infected lung tissue at the four time
points of infection. This procedure was chosen to avoid
introducing noise by comparing the in vivo data to an in vitro
grown bacterial control culture. By two-way ANOVA analysis
using the software R (http://www.r-project.org/), 250 open
reading frames (ORFs) were identified as significantly
(P<2×10⁻²) differentially expressed during the first 48 h of
infection. These 250 open reading frames corresponded to 237
unique genes (Table S3). With very few exceptions, most of these
genes displayed a steady decline in expression from 6 h to 48 h
p.i. It is reasonable to assume that the observed changes in gene
expression during the first 48 h of infection were related to the
changes induced by the bacteria entering the host; but without an
in vivo expression value for time zero of the infection (not
obtainable due to technical limitations), we cannot substantiate
this hypothesis. It has previously been shown, however, that
bacterial gene expression in response to environmental changes
happens very rapidly and mainly through gene activation [5,23].
A possible explanation for the observed differential expression,
where genes are induced early and then gradually decline in
expression, is that it may reflect the gradual adaption of A.
pleuropneumoniae to the new environmental conditions; possibly
characterized by the gradual deterioration of the host.

Because no suitable reference could be established for this
experimental set-up, important virulence genes might be over-
looked if these were constitutively expressed during infection. We
therefore included the constitutively most highly expressed genes
in the analysis. To avoid problems with background noise and to
keep the number of genes under investigation at a manageable
size, we selected a cut off value of mean log²≥13 (SD<0.5),
resulting in 133 ORFs which were the constitutively most highly
expressed genes (Table S4).

On the basis of clusters of orthologous groups (COGs)
classifications, categories that were overrepresented in the
differentially expressed gene set relative to their representation in
the A. pleuropneumoniae genome overall [24], were “energy
production and conversion”, “carbohydrate transport and metabol-
ism”, “post translational modification, protein turnover and
chaperones”, “amino acid transport and metabolism”, “inorganic
ion transport and metabolism”, “cell motility” and “unknown
functions” (Figure 2A). Ribosomal proteins or those involved in
translation, and to a lesser degree transcription, dominated the
group of constitutively highly expressed genes, both with regards to numbers of genes (31.5%) and level of expression (between log2 of 13.9 and 15.2). The high expression of ribosomal genes indicated a high growth rate in vivo (Figure 2B). Likewise overrepresented among the constitutively highly regulated genes were the functional groups “cell wall/membrane biogenesis” and “intracellular trafficking and secretion” (Figure 2B).

Comparison with other expression studies

Although direct comparison was complicated by differences in experimental designs, we cross-referenced our findings with recent expression studies of A. pleuropneumoniae [11,16–18,20–22] and another member of the Pasteurellaceae family, Haemophilus influenzae [25] (Table S5). Around 38% of the differentially regulated genes and 43% of the constitutively highly expressed genes, identified in the present study, had previously been identified as being differentially regulated during the infectious process, or during biofilm formation.

Adhesion and competence

A. pleuropneumoniae enters the airways after inhalation as an aerosol and colonizes the host by binding to mucus, proteins and host cells in the distal parts of the lung. This ability to adhere to host cells or surfaces is a vital part of a successful bacterial invasion [12,14]. Not surprisingly, we saw differential regulation of the type IV pili genes (apfAB), most likely induced by contact with lower respiratory tract epithelial cells thereby promoting adherence to these cells [11,26]. Two of the constitutively highly expressed genes, csgG and tufB, were also potential participants in the adhesion process. CsgG encodes a component in the production of long thin aggregative fimbiae (curli) with adhesive properties [27]. CsgG had previously been observed to be up-regulated in vivo in pig lung during the acute phase of disease and during biofilm formation [11,22]. Interestingly, the curli protein assembly was identified as a potent immunogenic protein in Haemophilus parasuis, the cause of Glässer’s disease in pigs [28]. The elongation factor tufB, has also previously been identified as a potential virulence factor, and is possibly involved in fibronectin binding [18].

In Vivo Transcription of A. pleuropneumoniae

Metabolic adaptations to in vivo conditions in the porcine lung

The results indicated that at 6 h p.i. A. pleuropneumoniae were encountering anaerobic conditions in the porcine lung. Table 1 lists the 32 genes involved in anaerobic metabolism that were displaying variations in gene expression during acute infection. Differential expression of the reductases, torYZ, dmsA and mfrABCEFG, suggested that A. pleuropneumoniae was using trimethylamine oxide, dimethyl sulfoxide and nitrite as terminal electron acceptors in anaerobic respiration [31]. DmsA has previously been demonstrated to have an effect on A. pleuropneumoniae virulence [32]. Among the significantly regulated genes were also the Ni/Fe cofactor dependent hydrogenases (hyaABD, hybAB), which catalyze the production and consumption of hydrogen gas.

Additionally, genes of the Na+ pump, oxaloacetate decarboxylase (nadAB) were differentially regulated over time while the Na+ exporting NADH dehydrogenase (mfrABCEFG) genes were constantly highly up-regulated.

The largest functional group of differentially regulated genes (14.5%) (Figure 2A) was predicted to be involved in carbohydrate transport and metabolism, which indicated a major shift in utilization of carbon sources for A. pleuropneumoniae when colonizing the host. Our results agreed with previous observations that genes responsible for transport and anaerobic metabolism of maltose (mal) and ascorbate (ula), respectively, were important in the acute...
phase of infection [11]. But, in addition, genes involved in uptake and metabolism of xylose (xyIAFGH) and galactose (galK, mgIABC) exhibited differential regulation over time. Also, two anaerobic pathways for glycerol dissemination (glp and dha) were significantly regulated in vivo. Both lead to the formation of dihydroxyacetone phosphate (DHAP), an intermediate of glycolysis [33]. Glycerol is an essential precursor for the synthesis of lipids and seems to be an important carbon/energy source for pathogenic bacteria [33].

The differential expression of methylglyoxal synthase, encoded by the mgIAC gene, could be a sign that A. pleuropneumoniae is actually experiencing carbon excess in early phase of infection. This bypass system produces methylglyoxal (MG) from the excess supply of DHAP [34,35]. MG is an extremely toxic electrophile, and the bacteria must therefore, rather paradoxically, protect itself against its own product. In E. coli, the principal route of MG detoxification is the glutathione-dependent glyoxalase system consisting of two enzymes glxI (glxB) and glxII (glgB) [34,36]. The glutathione conjugates activate the potassium efflux genes kefB and kefC, which lead to a lowering of the intracellular pH of the bacterial cell and protection against the toxic effects of MG [34]. While glxI and glxII appeared to be active at the same level throughout the trial, the kefB and kefC genes were differentially expressed during infection. KefB and KefC have also earlier been found regulated in A. pleuropneumoniae isolated from necrotic porcine lung tissue [18].

Few in vivo regulated genes involved in iron acquisition were found. We observed differential regulation of fnbA, which is part of an operon encoding proteins involved in uptake of exogenously supplied siderophores [37] and afuAB, constituting a periplasmic protein-dependent ABC-type Fe^{3+} transport system [38]. For microorganisms trying to colonize the mucosal surfaces of their host, haemin is a potentially valuable source of iron [39] and we also recorded differential regulation of hmuV, encoding a possible hemin ABC superfamily ATP binding cassette transporter. This gene has been reported not to be present in the commensal strains of Pasteurellaceae [40].

Cell wall metabolism

Synthesis of products related to cell wall/membrane biogenesis appears to be in high demand in early infection as this functional group was overrepresented among constitutively highly regulated genes (Figure 2B). Table 2 summarizes the 32 differentially or constitutively highly expressed genes with putative or known functions in cell wall/membrane biogenesis. A key enzyme in the biosynthesis of lipopolysaccharide (LPS), CMP-Kdo synthetase (kdsB), was differentially regulated over time. The LPS are some of the major surface components that interact directly with factors in the host environment and play a vital role in the infectious process [41] and the kdsB enzyme may constitute a possible target for the development of new antimicrobial agents against Gram-negative bacteria [42].

Likewise, genes from the peptidoglycan biosynthetic pathway (mruD) and mruI were differentially expressed in the initial stages of infection, along with bgB, a putative effector of murin hydrodase. Murin hydrodases are needed in order to expand the cell wall during bacterial growth and may therefore be important factors in determining the course of infection [43].

The enterobacterial common antigen (ECA) is a glycolipid present in the outer membrane in Gram-negative enteric bacteria. Genes responsible for the biosynthesis of ECA, (eevBCDE) were differentially expressed in this study. Probably belonging to the same operon and also differentially expressed over time was the O-antigen translocase, wzaE. The genes wcaABC were required for synthesis of lipid I and lipid II, while wscE was involved in lipid III synthesis. Lipid III is transported across the membrane via the wzc translocase [44]. We were not able to find other studies describing in vivo or in vitro up-regulation of ECA in A. pleuropneumoniae. Although present in all Gram-negative enteric bacteria, the function of ECA remains to be established.

It has previously been demonstrated that both lipoprotein E (ompP4) and the outer membrane protein P5 (ompP5/ompA) play active parts in the pathogenesis of H. influenzae [45,46]. In the current study, ompP4 was differentially regulated while the two ompP5 genes (APL_1421 and APL_1832), were constitutively highly expressed.

Stress response

The classical chaperones, Hsp70 (dnaK), Hsp40 (dnaJ) and dijA, and the periplasmic stress sensor, dsgX, were found to be constitutively highly expressed and may be important for bacterial survival within macrophages. Hsp70 and its co-chaperones are the most potent cellular defenses against environmental insults [47]. DnaK was reported as immunoreactive in convalescent sera from pigs naturally infected with A. pleuropneumoniae [48]. We observed in vivo activation of oxidative stress resistance mechanisms, represented by the two genes coding for thiol peroxidase (fps) and cytochrome c peroxidase (cp), respectively, both involved in protecting the bacteria against hydrogen peroxide [49]. In A. pleuropneumoniae grown in bronchoalveolar fluid, cp has earlier been found to be among the most highly up-regulated genes [21]. Also, it has been reported that the lipid hydroperoxide peroxidase, encoded by fps, protects Salmonella enterica from hydrogen peroxide stress in vitro and facilitates intracellular growth [50].

In the lungs, copper concentrations have been shown to increase during infection and inflammation [51]. As high concentrations of copper are toxic, bacteria have developed a number of mechanisms for dealing with excess concentrations of this metal. Efflux mechanisms include the ubiquitous copA1/copB1-type ATPase transporters [51]. We noticed significant regulation of copA (APL_1265) and the putative cation transport ATPase, most likely involved in copper detoxification (APL_1264). CopA has earlier been found to be important for survival in necrotic porcine lung tissue [19].

Urease activity may increase intracellular survival and impair macrophage function through the production of ammonia, which inhibits phagosome-lysosome fusion in macrophages [12,52,53]. The genes ureAGE were up-regulated in A. pleuropneumoniae during biofilm formation in vivo [22], but to our knowledge, this is the first report of significant regulation of urease genes (ureADEG) during in vivo infection. We did not, however, observe any differential regulation of the putative nickel and cobalt periplasmic permease system (cbiKLMQO) upstream of the urease cluster which appears to be required for urease activity in this bacterium [54]. As more than one of the biological systems, which requires Ni^{2+} for activity, were significantly regulated in this study (urease, NiFe hydrogenases), it is unclear why no nickel transport proteins appear to be regulated in vivo. A. pleuropneumoniae may harbor mechanisms of nickel uptake that have yet to be identified, similar to many other
Table 1. Differentially expressed \textit{A. pleuropneumoniae} genes involved in anaerobic metabolism.

<table>
<thead>
<tr>
<th>Gene designation</th>
<th>Locus no.*</th>
<th>Annotation</th>
<th>Functional group$^b$</th>
<th>Log$_{2}$ mean expression 6 h (n = 21)</th>
<th>Log$_{2}$ mean expression 12 h (n = 15)</th>
<th>Log$_{2}$ mean expression 24 h (n = 18)</th>
<th>Log$_{2}$ mean expression 48 h (n = 21)</th>
<th>P-value (differential expression)</th>
</tr>
</thead>
<tbody>
<tr>
<td>nrfC*</td>
<td>APL_0102</td>
<td>Nitrate reductase</td>
<td>C</td>
<td>12.41</td>
<td>12.47</td>
<td>11.19</td>
<td>10.92</td>
<td>1.20E-09</td>
</tr>
<tr>
<td>glpA</td>
<td>APL_0379</td>
<td>Sn-glycerol-3-phosphate dehydrogenase subunit A</td>
<td>C</td>
<td>11.18</td>
<td>10.93</td>
<td>10.48</td>
<td>9.94</td>
<td>1.96E-13</td>
</tr>
<tr>
<td>torZ*</td>
<td>APL_0688</td>
<td>Trimethylamine-N-oxide reductase precursor</td>
<td>C</td>
<td>12.73</td>
<td>12.13</td>
<td>11.31</td>
<td>10.54</td>
<td>1.21E-16</td>
</tr>
<tr>
<td>torY</td>
<td>APL_0689</td>
<td>Cytochrome c-type protein</td>
<td>C</td>
<td>12.66</td>
<td>12.13</td>
<td>11.23</td>
<td>10.44</td>
<td>2.82E-15</td>
</tr>
<tr>
<td>dccuC*</td>
<td>APL_0870</td>
<td>Putative C4-dicarboxylate transporter</td>
<td>C</td>
<td>11.21</td>
<td>11.04</td>
<td>10.12</td>
<td>9.46</td>
<td>6.39E-14</td>
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<tr>
<td>hydA*</td>
<td>APL_1331</td>
<td>Hydrogenase 2 small subunit</td>
<td>C</td>
<td>10.03</td>
<td>9.15</td>
<td>8.3</td>
<td>7.82</td>
<td>2.55E-22</td>
</tr>
<tr>
<td>hybA</td>
<td>APL_1332</td>
<td>Hydrogenase 2 protein</td>
<td>C</td>
<td>11.10</td>
<td>10.26</td>
<td>9.26</td>
<td>8.81</td>
<td>1.75E-21</td>
</tr>
<tr>
<td>hybB*</td>
<td>APL_1333</td>
<td>Putative Ni/Fe-hydrogenase 2 b-type cytochrome subunit</td>
<td>C</td>
<td>12.03</td>
<td>11.64</td>
<td>10.39</td>
<td>9.72</td>
<td>6.76E-14</td>
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<tr>
<td>hydB*</td>
<td>APL_1334</td>
<td>Hydrogenase 2 large chain</td>
<td>C</td>
<td>12.24</td>
<td>11.69</td>
<td>10.4</td>
<td>9.94</td>
<td>2.46E-15</td>
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<tr>
<td>hydD</td>
<td>APL_1335</td>
<td>Hydrogenase 2 maturation protease</td>
<td>C</td>
<td>12.46</td>
<td>11.95</td>
<td>11.18</td>
<td>10.8</td>
<td>3.99E-18</td>
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<tr>
<td>oadA</td>
<td>APL_1376</td>
<td>Oxaloacetate decarboxylase alpha chain</td>
<td>C</td>
<td>11.25</td>
<td>11.1</td>
<td>10.17</td>
<td>9.78</td>
<td>1.59E-09</td>
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<tr>
<td>oadB</td>
<td>APL_1377</td>
<td>Oxaloacetate decarboxylase beta chain</td>
<td>C</td>
<td>11.45</td>
<td>11.42</td>
<td>10.54</td>
<td>10.01</td>
<td>1.46E-09</td>
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<tr>
<td>dmsA*</td>
<td>APL_1674</td>
<td>Anaerobic dimethyl sulfoxide reductase chain A precursor</td>
<td>C</td>
<td>13.17</td>
<td>13.32</td>
<td>12.3</td>
<td>11.7</td>
<td>1.34E-09</td>
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<tr>
<td>dhaM</td>
<td>APL_0081</td>
<td>PTS-dependent dihydroxyacetone kinase. phosphotransferase subunit</td>
<td>S</td>
<td>11.49</td>
<td>11.14</td>
<td>10.31</td>
<td>9.94</td>
<td>1.07E-14</td>
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<tr>
<td>dhaL</td>
<td>APL_0082</td>
<td>PTS-dependent dihydroxyacetone kinase. ADP-binding subunit</td>
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<td>12.55</td>
<td>12.3</td>
<td>10.86</td>
<td>10.42</td>
<td>4.31E-14</td>
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<tr>
<td>dhaK</td>
<td>APL_0083</td>
<td>PTS-dependent dihydroxyacetone kinase. dihydroxyacetone-binding subunit</td>
<td>G</td>
<td>11.37</td>
<td>11.1</td>
<td>9.94</td>
<td>9.08</td>
<td>1.29E-17</td>
</tr>
<tr>
<td>mdlK*</td>
<td>APL_1236</td>
<td>Maltose/maltodextrin import ATP-binding protein</td>
<td>G</td>
<td>10.8</td>
<td>10.61</td>
<td>9.42</td>
<td>8.72</td>
<td>1.08E-11</td>
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<tr>
<td>mdlQ*</td>
<td>APL_1240</td>
<td>4-alpha-glucanotransferase</td>
<td>G</td>
<td>12.57</td>
<td>11.99</td>
<td>11.16</td>
<td>10.62</td>
<td>1.22E-09</td>
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<td>ulaD*</td>
<td>APL_1698</td>
<td>Probable 3-keto-L-gulonate-6-phosphate decarboxylase</td>
<td>G</td>
<td>11.92</td>
<td>10.91</td>
<td>10.38</td>
<td>10.27</td>
<td>1.05E-10</td>
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<td>ulaC*</td>
<td>APL_1699</td>
<td>Ascorbate-specific phosphotransferase enzyme IIIA component</td>
<td>G</td>
<td>9.83</td>
<td>9.02</td>
<td>8.88</td>
<td>8.74</td>
<td>9.09E-14</td>
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<td>ulaG*</td>
<td>APL_1701</td>
<td>L-ascorbate-6-phosphate lactonase UlaG-like protein</td>
<td>R</td>
<td>12.45</td>
<td>11.92</td>
<td>11.37</td>
<td>11.03</td>
<td>9.03E-11</td>
</tr>
<tr>
<td>ulaR</td>
<td>APL_1702</td>
<td>HTH-type transcriptional regulator</td>
<td>G</td>
<td>11.3</td>
<td>10.74</td>
<td>9.68</td>
<td>9.36</td>
<td>4.27E-13</td>
</tr>
<tr>
<td>ulaA</td>
<td>APL_1714</td>
<td>Ascorbate-specific permease UGA component</td>
<td>S</td>
<td>11.35</td>
<td>10.67</td>
<td>9.57</td>
<td>8.48</td>
<td>6.02E-18</td>
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<tr>
<td>chuW*</td>
<td>APL_1523</td>
<td>Coproporphyrinogen III oxidase</td>
<td>H</td>
<td>11.28</td>
<td>10.7</td>
<td>9.86</td>
<td>9.36</td>
<td>1.45E-13</td>
</tr>
<tr>
<td>nrfG*</td>
<td>APL_1067</td>
<td>Formate-dependent nitrite reductase complex</td>
<td>O</td>
<td>10.98</td>
<td>10.36</td>
<td>9.35</td>
<td>8.79</td>
<td>4.79E-14</td>
</tr>
<tr>
<td>nrfF</td>
<td>APL_1068</td>
<td>Formate-dependent nitrite reductase complex</td>
<td>O</td>
<td>9.39</td>
<td>9.08</td>
<td>8.66</td>
<td>8.33</td>
<td>8.67E-13</td>
</tr>
<tr>
<td>nrfE</td>
<td>APL_1052</td>
<td>Cytochrome c-type biogenesis protein</td>
<td>O</td>
<td>11.23</td>
<td>11.2</td>
<td>10.43</td>
<td>9.93</td>
<td>2.94E-10</td>
</tr>
<tr>
<td>nrfA</td>
<td>APL_0100</td>
<td>Cytochrome c-552</td>
<td>P</td>
<td>13.64</td>
<td>13.56</td>
<td>12.22</td>
<td>12</td>
<td>1.10E-10</td>
</tr>
<tr>
<td>nrfB*</td>
<td>APL_0101</td>
<td>Cytochrome c-type protein</td>
<td>P</td>
<td>12.54</td>
<td>12.4</td>
<td>10.81</td>
<td>10.43</td>
<td>2.60E-10</td>
</tr>
</tbody>
</table>
bacteria which use nickel without possessing homologues of the known nickel/cobalt transporters [53].

Evading host immune response

Our results indicated that sialic acid metabolism could be of importance for the survival and persistence of A. pleuropneumoniae in the porcine lung. Sialic acids are the terminal sugars of the host cellular glyocalyx and therefore one of the first substances that the microbe encounters when it enters the host [56]. Sialic acid is an attractive nutritional source for microbes that associate with vertebrates [56]. In this study, five putatively co-regulated genes related to sialic acid metabolism, were differentially regulated. These were neuA, nanEA and nagBA. Activation of the nan operon depends on the availability of sialic acid in the environment. NanA cleaves sialic acid to produce N-Acetyl-D-mannosamine and pyruvate. N-Acetyl-D-mannosamine is converted to fructose-6-P and glucosamine-6-P by the concerted action of nanEK and nagAB [56].

Intracellular trafficking and secretion

The general secretion pathway (sec-pathway) is the major route of protein translocation across the cytoplasmic membrane in bacteria [57]. We observed constitutively high in vivo expression of secA, secB, secD, secF and yajC—all genes encoding components of the predicted sec translocation system. Also genes from the sec independent twin-arginine translocation (Tat) export pathway were differentially (tatA) or constitutively highly (tatB) expressed in this investigation. The Tat pathway is utilized by bacteria to export pre-folded proteins, in particular cofactor containing redox enzymes, across the bacterial inner membrane into the periplasmic compartment [58]. In H. influenzae, the tatA and tatB genes were both earlier found to be among the genes imperative for bacterial survival in a murine lung model [25]. In E. coli, Tat deficient mutants displayed phenotypic characteristics consistent with an outer membrane defect [59]. Proteins targeted for export by the Tat pathway usually possess a twin arginine signal motif, ([S/T]RRXFLK) in the N-terminus [60]. In the present study, the Tat signal motif was identified in 7 differentially regulated A. pleuropneumoniae genes (nrfC, torZ, hyaA, hybD, dmsA, cpdB and ywbN) by the signal prediction server TatFind (http://signalfind.org/tatfind.html) [61]. Five of these genes, nrfC, torZ, hyaA, hybD and dmsA, encode proteins belonging to the energy production and conversion functional group and are involved in anaerobic growth.

In vivo expression of exotoxins

It is well established that the secreted pore-forming RTX exotoxins are among the most important virulence factors in A. pleuropneumoniae, directly involved in causing necrotic lesions of the target organs [62]. A. pleuropneumoniae serotype 2 and 6 secrete the exotoxins apxII, apxIII and apxVI. In this study, only apxA (log2 = 13.94; SD = 0.34) was above the set threshold for constitutively highly expressed genes. The toxin genes apxA (log2 = 12.94; SD = 0.85) and apxIA (log2 = 11.14; SD = 0.30) were, however, both actively transcribed during the study period. The RTX toxin, ApxIV, is not expressed in vitro but activates high levels of serum antibodies during infection [63,64]. The apxIVA gene and has previously been demonstrated to be specifically induced during infection [11].

Global regulation in A. pleuropneumoniae

As the only global regulator, the ferric-uptake regulator protein (Fur) was constitutively highly expressed throughout the experiment (Mean log2 = 13.40; SD = 0.46). Fur plays a key role in
Table 2. Differentially or highly expressed *A. pleuropneumoniae* genes with expected or putative functions in cell wall/membrane biogenesis.

<table>
<thead>
<tr>
<th>Gene designation</th>
<th>Locus no.*</th>
<th>Annotation</th>
<th>Functional group</th>
<th>Log2 mean expression 6 h (n = 21)</th>
<th>Log2 mean expression 12 h (n = 15)</th>
<th>Log2 mean expression 24 h (n = 18)</th>
<th>Log2 mean expression 48 h (n = 21)</th>
<th>P-value (differential expression)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>rsmH</em></td>
<td>APL_0010</td>
<td>Ribosomal RNA small subunit methyltransferase</td>
<td>H</td>
<td>11.39</td>
<td>11.18</td>
<td>10.71</td>
<td>10.45</td>
<td>1.30E-11</td>
</tr>
<tr>
<td><em>murD</em></td>
<td>APL_0016</td>
<td>UDP-N-acetylmuramylasaline-D-glutamate ligase</td>
<td>M</td>
<td>11.63</td>
<td>11.32</td>
<td>10.69</td>
<td>10.35</td>
<td>3.26E-11</td>
</tr>
<tr>
<td><em>ftsO</em></td>
<td>APL_0021</td>
<td>Cell division protein</td>
<td>M</td>
<td>13.23</td>
<td>13.40</td>
<td>12.96</td>
<td>13.00</td>
<td>NS</td>
</tr>
<tr>
<td><em>lpC</em></td>
<td>APL_0024</td>
<td>UDP-3-O-(3-hydroxymyristoyl) N-acetylglucosamine deacetylase</td>
<td>M</td>
<td>13.41</td>
<td>13.36</td>
<td>13.02</td>
<td>13.09</td>
<td>NS</td>
</tr>
<tr>
<td><em>kdb</em></td>
<td>APL_0085</td>
<td>3-deoxy-manno-octulosonate cytidylyltransferase</td>
<td>M</td>
<td>10.82</td>
<td>10.27</td>
<td>9.50</td>
<td>9.14</td>
<td>8.10E-10</td>
</tr>
<tr>
<td><em>prc</em></td>
<td>APL_0120</td>
<td>Carboxy-terminal protease</td>
<td>M</td>
<td>13.21</td>
<td>13.29</td>
<td>12.97</td>
<td>13.09</td>
<td>NS</td>
</tr>
<tr>
<td><em>csgG</em></td>
<td>APL_0220</td>
<td>Putative lipoprotein</td>
<td>M</td>
<td>13.91</td>
<td>14.07</td>
<td>14.05</td>
<td>14.18</td>
<td>NS</td>
</tr>
<tr>
<td>APL_0221</td>
<td>APL_0221</td>
<td>Putative lipoprotein, periplasmic protein</td>
<td>S</td>
<td>14.35</td>
<td>14.46</td>
<td>14.46</td>
<td>14.58</td>
<td>NS</td>
</tr>
<tr>
<td>APL_0234</td>
<td>APL_0234</td>
<td>23S rRNA pseudouridine synthase</td>
<td>D</td>
<td>11.92</td>
<td>11.36</td>
<td>10.55</td>
<td>10.33</td>
<td>1.53E-11</td>
</tr>
<tr>
<td><em>toIA</em></td>
<td>APL_0302</td>
<td>Cell envelope integrity inner membrane protein</td>
<td>M</td>
<td>13.24</td>
<td>13.27</td>
<td>13.07</td>
<td>13.44</td>
<td>NS</td>
</tr>
<tr>
<td><em>nlpc</em></td>
<td>APL_0359</td>
<td>Putative lipoprotein</td>
<td>M</td>
<td>13.31</td>
<td>13.41</td>
<td>13.20</td>
<td>13.20</td>
<td>NS</td>
</tr>
<tr>
<td><em>ompP4</em>*</td>
<td>APL_0389</td>
<td>Lipoprotein E</td>
<td>R</td>
<td>12.46</td>
<td>11.71</td>
<td>10.77</td>
<td>9.98</td>
<td>4.29E-14</td>
</tr>
<tr>
<td><em>macA</em></td>
<td>APL_0391</td>
<td>Probable malonil-specific efflux protein</td>
<td>M</td>
<td>11.74</td>
<td>11.44</td>
<td>10.52</td>
<td>10.49</td>
<td>4.45E-10</td>
</tr>
<tr>
<td><em>nlpl</em></td>
<td>APL_0576</td>
<td>Lipoprotein Nlpl-like</td>
<td>R</td>
<td>13.89</td>
<td>14.06</td>
<td>14.06</td>
<td>14.15</td>
<td>NS</td>
</tr>
<tr>
<td><em>acrA</em></td>
<td>APL_0586</td>
<td>Putative RND efflux membrane fusion protein</td>
<td>M</td>
<td>13.27</td>
<td>13.43</td>
<td>13.32</td>
<td>13.45</td>
<td>NS</td>
</tr>
<tr>
<td><em>igB</em></td>
<td>APL_0779</td>
<td>Putative effector of murein hydrolase</td>
<td>M</td>
<td>10.63</td>
<td>9.83</td>
<td>9.16</td>
<td>8.40</td>
<td>7.16E-15</td>
</tr>
<tr>
<td><em>ompW</em></td>
<td>APL_1086</td>
<td>Outer membrane protein W</td>
<td>M</td>
<td>12.07</td>
<td>11.88</td>
<td>10.80</td>
<td>10.17</td>
<td>7.01E-12</td>
</tr>
<tr>
<td>APL_1121</td>
<td>APL_1121</td>
<td>Putative lipoprotein</td>
<td>R</td>
<td>13.70</td>
<td>13.66</td>
<td>13.38</td>
<td>13.39</td>
<td>NS</td>
</tr>
<tr>
<td><em>wecE</em></td>
<td>APL_1549</td>
<td>TDP-4-keto-6-deoxy-D-glucose transaminase</td>
<td>M</td>
<td>10.13</td>
<td>9.71</td>
<td>8.98</td>
<td>8.45</td>
<td>1.07E-11</td>
</tr>
<tr>
<td><em>wecD</em></td>
<td>APL_1550</td>
<td>Putative TDP-D-fucosyltransferase</td>
<td>M</td>
<td>9.71</td>
<td>9.05</td>
<td>8.58</td>
<td>8.00</td>
<td>6.51E-16</td>
</tr>
<tr>
<td><em>wecC</em></td>
<td>APL_1551</td>
<td>UDP-N-acetyl-D-mannosamine dehydrogenase</td>
<td>M</td>
<td>10.45</td>
<td>10.03</td>
<td>9.33</td>
<td>8.69</td>
<td>8.68E-15</td>
</tr>
<tr>
<td><em>wecB</em></td>
<td>APL_1552</td>
<td>UDP-N-acetylgulosamine 2-epimerase</td>
<td>M</td>
<td>10.63</td>
<td>10.28</td>
<td>9.37</td>
<td>8.91</td>
<td>1.33E-11</td>
</tr>
<tr>
<td>APL_1597**</td>
<td>APL_1597</td>
<td>Rare lipoprotein A</td>
<td>M</td>
<td>13.93</td>
<td>13.80</td>
<td>13.40</td>
<td>13.39</td>
<td>NS</td>
</tr>
<tr>
<td><em>glmS</em></td>
<td>APL_1631</td>
<td>Glucosamine-fructose-6-phosphate aminotransferase</td>
<td>M</td>
<td>12.74</td>
<td>13.12</td>
<td>13.11</td>
<td>13.19</td>
<td>NS</td>
</tr>
<tr>
<td><em>mrc</em>*</td>
<td>APL_1741</td>
<td>Membrane-bound lytic murein transglycosylase C</td>
<td>M</td>
<td>13.27</td>
<td>13.23</td>
<td>12.93</td>
<td>12.85</td>
<td>NS</td>
</tr>
<tr>
<td><em>murI</em></td>
<td>APL_1841</td>
<td>Glutamate racemase</td>
<td>M</td>
<td>11.08</td>
<td>10.65</td>
<td>10.13</td>
<td>9.77</td>
<td>9.54E-13</td>
</tr>
</tbody>
</table>

*Genes previously identified as being relevant for *A. pleuropneumoniae* infection or biofilm formation [11,17,18,20–22].
**Haemophilus influenzae** genes required in the lung determined in a murine pulmonary model of infection [25].

*Locus numbers from *A. pleuropneumoniae* serotype 5 (L28).

*Function of genes according to Clusters of Orthologous Groups of proteins (COGs). M: cell wall/membrane biogenesis; R: general function prediction, only; S: function unknown.

P-values are only included for the genes that were differentially expressed over time, the constitutively highly expressed genes were of course not significant (NS). doi:10.1371/journal.pone.0035549.t002
controlling iron homeostasis at the level of transcription by sensing intracellular iron levels and adjusting gene expression accordingly [63,66]. This protein directly regulates most iron-acquisition genes in a negative fashion by blocking their transcription when intracellular iron is at an acceptable level. It has now become clear that Fur, through a small (s)RNA named RyhB, also indirectly acts as a transcriptional activator switching on genes, many of which encode iron-rich respiratory complexes [67]. In *E. coli*, a large group of energy metabolism genes was found to be iron and Fur induced, including genes involved in oxidative stress response and virulence [63,68]. The general pattern of gene expression observed in this study was in accordance with a Fur regulator with ferrous iron bound. Firstly, only a few iron acquisition genes were differentially expressed, which indicated that *A. pleuropneumoniae* was not encountering iron-restriction during this period of the *in vivo* infection. Secondly, a number of genes coding for metabolic enzymes dependent on Fe-S clusters or other iron cofactors (e.g. *dmsA*, *hpaABD* and *torV*) appeared to be actively transcribed and may have been indirectly activated by Fur through RyhB [67].

### Discussion

Monitoring bacterial expression *in situ* during infection is an opportunity to gain unique insight into the molecular mechanisms of host-pathogen interactions, as the *in vivo* expression profile of the microbial invader can also serve as indicator of the host microenvironment. To study this process closer in the porcine pathogen *A. pleuropneumoniae*, we undertook the first large scale time-course study of this bacterium’s *in vivo* transcriptome during the first 48 h of infection. *A. pleuropneumoniae* was able to establish severe infection in the lungs of the porcine host within 6 h. However, 17 of the 48 pigs did not develop infection, three pigs died before tissue sampling and three samples were discarded during analysis. This resulted in the loss of balance for the data and presents a two-fold challenge in the interpretation. First, the final 9 and 16 samples of serotype 6 and 2, respectively, were biased in that serotype 6 samples were more prevalent, particularly for the 6 h time point. Second, as a consequence of the first, time and serotype could no longer be assumed to be independent. For these reasons, we only considered the time factor and abstained from drawing any conclusions based on serotype. The inclusion of the serotype factor in the 2-way ANOVA should thus be seen as an endeavor to reduce the potential bias this factor might otherwise introduce to the analysis.

From the 25 samples under analysis, many of the differentially regulated genes were not highly expressed at any time point during the monitored period. The differences in expression were, nevertheless, highly significant, which strongly indicated that they may represent factors required by *A. pleuropneumoniae* for the disease process, even if they are not required at great abundance. We are convinced that by identifying genes that are differentially expressed *in vivo* in response to changes in environmental parameters rather than applying an *in vitro* grown culture as reference condition, subtle changes in gene expression were detected that would otherwise have been missed. For example, we were able to see a distinct shift to anaerobic metabolism (Table 1) which was not detected, when comparing *in vitro* results to *in vitro* culture conditions [11]. The data included many genes previously reported to be implicated in virulence of *A. pleuropneumoniae* and other pathogenic Gram-negative bacteria. But in addition to adding further weight to previous observations of *A. pleuropneumoniae* pathogenesis, this investigation also revealed potential new strategies for adapting to the host environment.

### Cross-references to other expression studies

The results most similar to our investigation were obtained from a gene expression analysis of *A. pleuropneumoniae* (serotype 5b, strain L20), isolated from one naturally infected pig during the exponential phase of infection [11]. In this study, 150 differentially expressed genes were identified *in vivo*, when compared to exponentially growing planktonic cultures in rich laboratory media [11]. Of these 150 genes, 38 were also detected in our study (24 differentially expressed/14 constitutively highly expressed). The genes we observed to be differentially regulated were up-regulated in the study of *A. pleuropneumoniae* serotype 5b; while the genes we identified as constitutively highly expressed were mostly down-regulated in that study (Table S5) [11]. Most likely these discrepancies reflected the difficulties of comparing studies with different experimental design; in this case, a study measuring differences between two growth conditions in *A. pleuropneumoniae* serotype 5b [11] versus the present study measuring changes in bacterial response over time in *A. pleuropneumoniae* serotype 2 and serotype 6.

Also included in the comparison, were microarray gene expression profiles of *A. pleuropneumoniae* exposed to bronchoalvolar fluid, attached to lung epithelial cells and during biofilm formation, respectively [20–22]. Both the transcriptome analysis of *A. pleuropneumoniae* exposed to bronchoalvolar fluid and our data suggested that the expression of genes involved in anaerobic energy generation and the synthesis of proteins involved in cell wall biogenesis were modulated in the early stages of infection [21]. We also cross-referenced our results to three other *in vivo* studies, identifying genes imperative for survival of *A. pleuropneumoniae*, in the host by STM and SCOTS, respectively [16–18], and against a study which used the method “high-throughput insertion tracking by deep sequencing” (HITS) for the identification of *H. influenzae* genes required for survival in a murine pulmonary model [25]. We identified 31 genes, which according to the various *in vivo* methods (STM, HITS, SCOTS), were important for bacterial survival in the host. Eight of these genes, e.g. the chaperone protein, *dutA*, and the anaerobic dimethyl sulfoxide reductase, *dmsA*, were found in *H. influenzae*, which illustrates that some common strategies for survival in the host are shared among members of Pasteurellaceae.

From the previous studies shown in Table S5, however, no clear consensus emerges—indeed, the majority of loci identified across all seven studies were found in no more than two. The observed variation in outcome probably reflects the differences in methodology and bacterial strains used.

### Global gene regulation during infection

Possibly sRNAs, may be influencing the global pattern of bacterial gene regulation during anaerobic conditions in the host [69]. In that aspect, the global iron regulator Fur may be interesting, as there is an emerging picture of Fur as an important regulator, either directly or indirectly, of global RNA expression in bacteria. Present evidence suggests that Fur plays a global role in basic bacterial physiology and has a considerably wider impact on gene expression, at least in some bacterial species, than originally perceived [66,70]. We also observed gene expression patterns *in vivo* which were consistent with active Fur-regulation, indicating that Fur could be governing functions influencing survival in the lung environment. Supporting this assumption is also the previous demonstration that a Fur mutant of *A. pleuropneumoniae* showed growth deficiencies *in vitro* and reduced virulence in an aerosol infection model [71].
Secretion systems

In Gram-negative bacteria, specialized protein secretion systems are essential for transport of virulence factors, mainly toxins, adhesins and proteases, across the two membranes and into the extracellular environment [72,73]. The Tat apparatus is well conserved among bacterial pathogens and appears to be involved in several virulence related traits such as iron uptake, anaerobic respiration, osmotic stress defense, copper homeostasis, motility and biofilm formation – all factors which are important for the pathogens ability to colonize and survive in the host [59,74]. Our results, identifying 7 genes whose products are putatively exported by the Tat-pathway, indicated that this system also could be of importance for A. pleuropneumoniae pathogenesis.

Metabolic adaptations to host environment

An interesting new observation in A. pleuropneumoniae expression during infection was the regulation of competence genes in early infection. This phenomenon has also been observed in vivo in other bacteria, such as Listeria monocytogenes and Streptococcus pneumoniae in animal models of infection [5,75]. Extracellular DNA is highly abundant in natural environments, for example in lung mucus, and the ability to consume this extracellular DNA, as a source of nutrition or to increase genetic fitness, may convey enhanced survival for the bacteria [76].

After successful attachment, A. pleuropneumoniae requires nutrients provided by the host to grow and cause disease. Generally, the supply of essential nutrients is limited in the lower respiratory tract. A. pleuropneumoniae can overcome this problem by the induction of lysis of host cells by secreted exotoxins, resulting in the release of nutrients into the environment [14]. The combined effect of rapid bacterial proliferation, exotoxins and host immune factors probably results in extensive tissue destruction and the formation of fibrino-hemorrhagic lesions quite early in the infectious process. Such lesions are likely to represent an anaerobic environment [77]. The observed amount of differentially expressed genes involved in anaerobic metabolism, clearly indicated that the pathogen was experiencing anaerobic growth conditions quite early in the infection (Table 1).

Also worth noticing—and to our knowledge—not previously reported in A. pleuropneumoniae during in vivo infection, was the differential regulation of the Na+ pump, oxaloacetate decarboxylase (oadAB), along with the simultaneous differential expression of the Na+(+)/H+(+) antiporter (nhaB), which play a major role in pH and Na+ homeostasis, this was a strong indication that Na+ pumps were of importance during infection. In H. influenzae, nhaB was among the genes required for growth and survival in a murine pulmonary model [25]; and up-regulation of nhaB and nhaA was also reported in A. pleuropneumoniae exposed to BALF [21]. Na+ gradient generation by decarboxylase-coupled ion transfer has only been identified in a limited number of (mostly) anaerobic bacteria, making it an exception rather than a rule in the microbial world [78]. Genes encoding primary Na+ pumps are found in the genomes of a number of phylogenetically diverse pathogenic bacteria. It is therefore quite possible that generation of a Na+ gradient is an important part of their membrane energetic, possibly constituting an alternative way of providing the bacteria with additional means of ATP synthesis, motility, and solute uptake which could improve its chances of colonization and survival in the host [78]. This study produced results which corroborate the findings of previous studies indicating that enzymes of anaerobic metabolism are essential for persistence of A. pleuropneumoniae in the host [79,80].

Coping with the host immune response

The initial interaction between A. pleuropneumoniae and the porcine host takes place on the epithelial lining of the respiratory tract, where the microbial intruder has to face the host’s first line of defense. The innate immune response is active against a broad spectrum of microbial pathogens and operates before an antigenic (adaptive) immune response has developed [84]. Alveolar macrophages and polymorphonuclear leukocytes constitute the major defense mechanism of the distal airways against invading microorganisms [85]. In these phagocytes, antimicrobial peptides, hydrolytic enzymes or reactive oxygen intermediates are released [84]. Both alveolar macrophages and polymorphonuclear leukocytes are able to phagocytose A. pleuropneumoniae, but only polymorphonuclear leukocytes can effectively kill the pathogen [85]. A. pleuropneumoniae is able to survive for more than 90 min within alveolar macrophages, during which time liberation of RTX toxins from the bacteria can destroy these host immune cells. This capacity to survive within macrophages may be due to several factors such as capsule, LPS, stress proteins and ammonia [12].

Host-pathogen interaction was reflected in the active remodeling of the bacterial envelope through activation of genes responsible for cell wall components (Table 2). The cell wall and membrane may provide important protection against cell surface damaging factors of the host environment. The resistance of A. pleuropneumoniae to complement cytotoxicity, for example, can mainly be attributed to the capsular polysaccharide (cps) and/or LPS [12]. We didn’t detect any significantly differential or high expression of the cps genes. In a previous investigation, the cps
genes were found to be down-regulated in A. pleuropneumoniae in vivo [11]. In early infection, the expression of a thick capsule may be disadvantageous for the bacterium because of its inhibitory effect on adherence [11,86]. This phenomenon has also been observed in other lung pathogens such as S. pneumoniae, where expression of a thinner capsule promotes binding to host tissue during initial stages of colonization [87].

In A. pleuropneumoniae, host contact may have induced significant regulation or high expression of genes coding for key enzymes in LPS biosynthesis (babD), the peptidoglycan biosynthetic pathway (nuuOr), lipoprotein E synthesis (ompP5), enterobacterial common antigen (ECA) (secBCDE) and ompP5 (ompD). In H. influenzae, lipoprotein E is essential for laminin uptake and the utilization of Nicotinamide adenine dinucleotide [45,88,89]. As A. pleuropneumoniae biotype-1 is also dependent on exogenous sources of Nicotinamide adenine dinucleotide for growth [90], lipoprotein E could also be relevant for the pathogenesis of this bacterium.

In non-typable H. influenzae (NTHI), OmpP5 binds specifically to a variety of receptors on the host cell membrane, including respiratory mucin and bronchial epithelial cells [91]. It is interesting to note, that in NTHI, OmpP5-derived peptides provide significant protection against homologous and heterologous NTHI challenge in chinchilla and rat models of otitis media [46]. Previously, APL_1421 has been reported to be expressed in necrotic porcine lung tissue by SCOTS [118], while an APL_1852 homolog was among the genes required by H. influenzae for survival in the murine lung [25]. Finally, a new study identified both OmpP5 proteins as immunoreactive in sera from swine naturally infected with A. pleuropneumoniae serotype 1 and in hyperimmune sera raised in an immunized rabbit [48]. This corresponds well with the findings in the current study, where both APL_1421 and APL_1852 were found constitutively highly expressed.

From the in vivo induction of a number of stress genes, we were able to make inferences concerning the stress factors A. pleuropneumoniae is facing in the host and the genes that were mobilized in order to survive in this harsh environment. Our results indicated that the pathogen encountered—and were able to cope with—host immune factors, e.g. reactive oxygen intermediates, produced by the alveolar macrophages as well as toxic concentrations of copper. Also, we observed significant differential expression of the genes ureaDEG, encoding urease subunits which may be involved in resistance to macrophage damage and bacterial chronic infection [52,53].

Sialic acid may function as an anti-recognition molecule, modifying the bacterial cell surface to mimic the host cell membrane, including respiratory mucin and bronchial epithelial cells [91]. It is interesting to note, that in NTHI, OmpP5-derived peptides provide significant protection against homologous and heterologous NTHI challenge in chinchilla and rat models of otitis media [46]. Previously, APL_1421 has been reported to be expressed in necrotic porcine lung tissue by SCOTS [118], while an APL_1852 homolog was among the genes required by H. influenzae for survival in the murine lung [25]. Finally, a new study identified both OmpP5 proteins as immunoreactive in sera from swine naturally infected with A. pleuropneumoniae serotype 1 and in hyperimmune sera raised in an immunized rabbit [48]. This corresponds well with the findings in the current study, where both APL_1421 and APL_1852 were found constitutively highly expressed.

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Sialic acid may function as an anti-recognition molecule, modifying the bacterial cell surface to mimic the host cell surface and subvert or inhibit host innate immunity [56,92]. This function has been implicated as a virulence factor in several bacterial species [56]. For example, LPS sialylation is a feature of several pathogenic members of the Pasteurellaceae, including H. ducreyi, H. influenzae, Histophilus somni and P. multocida [93]. The significant regulation of acylneuraminic cytidylyltransferase (neuA) leaves open the possibility that A. pleuropneumoniae could be using a mechanism of cell surface sialylation, called precursor scavenging. This method is also applied by H. influenzae, which, like A. pleuropneumoniae, lacks neuBC but has orthologues of neuD [92,94]. In H. influenzae, sialylated LPS glycoforms play a key role in pathogenicity of nontypeable variants which scavenge the essential precursors from the host during the infection shown in a chinchilla model of otitis media [92]. For P. multocida, sialylation appears to be necessary for systemic pasteurulosis, presumably by protecting the sialylated bacteria from innate host defense mechanisms [95]. Further investigations are necessary to clarify the potential influence of sialic acid cell surface modifications upon the virulence of A. pleuropneumoniae.

Concluding remarks

The outcome of a bacterial infection is determined by the complex interactions of multiple host and microbial factors. The physiochemical elements of this relationship are very difficult to reproduce under in vitro conditions. By monitoring the bacterial in vivo genomic expression during the first critical phase of infection in its natural host, we were able to derive new detailed information regarding host-pathogen interactions. Data presented here illustrated how A. pleuropneumoniae was able to adapt its metabolism to derive carbon and energy from an anaerobic environment and how the microbe was employing a broad range of strategies to evade and counteract the effects of the host immune response. Understanding the metabolic basis of bacterial pathogenesis may provide a rational basis for the development of new therapeutical strategies. Many new targets for future research have been uncovered and future phenotypic analysis will show if some of the potential virulence genes identified here may serve as new targets in drug and vaccine development.

Materials and Methods

Ethics statement

All animal procedures were approved by the Danish Animal Experiments Inspectorate under the Ministry of Justice (permit number: 2006/561-1106) and the animal experiments were conducted in strict accordance with their guidelines.

Bacterial strains and growth conditions

A. pleuropneumoniae serotype 2 (4226) and serotype 6 (7712640), both Danish field strains isolated from pigs with acute pleuropneumonia, were used for the infection studies. A. pleuropneumoniae was grown on PPLO agar plates (Difco) at 37°C overnight and subsequently resuspended in 0.9% NaCl and adjusted to a density corresponding to McFarland standard 1. This suspension was mixed 1:1 with Brain heart infusion broth (Difco) added 5% NAD and used for infection.

Infection studies

For A. pleuropneumoniae serotype 2 and 6, respectively, 24 8–10-week-old Danish specific pathogen free (SPF) piglets were infected via the intranasal route. Due to practical reasons, the infection experiments for each of the serotypes were performed on separate days. The animals received 2 ml of a bacterial suspension containing a total dose of 2 x 10⁸ CFU or 1 x 10⁹ CFU of serotype 2 or 6, respectively. Six animals were sacrificed 6 h, 12 h, 24 h and 48 h post infection, respectively. The animals were sedated with Zoletil ® (Virbac, Carros, France) and Narcoxyl ® Intervet MSD Animal Health (Ballerup, Denmark) and euthanized with pentobarbitol (Veterinærapoteket, University of Copenhagen, Denmark). Of the 48 pigs one was euthanized due to unrelated neck infection and another two succumbed to serotype 2 infections before sampling. In total 28 animals displayed visual lung lesions (Table S1). Immediately post mortem, infected lung tissue was isolated, cut into pieces smaller than 0.5 x 0.5 cm and preserved in RNAlater stabilization reagent (Ambion, Cambridgeshire, United Kingdom) at −20°C. Samples from the lungs were cultivated on PPLO agar (SSI, Copenhagen, Denmark) and Columbia agar plates (Oxoid, Greve, Denmark) with 5% calf blood added at 37°C in atmospheric air over night to re-isolate the inoculation strain and other bacteria present. Bacterial identification was done according to the standard procedures of the laboratory.
RNA isolation and reverse transcription

Total RNA was isolated from 100 to 300 mg lung tissue with visual lesions (3 samples from each animal). Prior to RNA extraction using the RNeasy Lipid kit (QIAGEN, Hilden, Germany), the tissue was finely chopped by scalpel, transferred to 5 ml Phenol/Quanidinium isothiocyanate lysis buffer (provided in the QIagen kit) in which it was divided further by a Tissue-Tearor, 985370-XL (BioSpec Products, Bartlesville, OK) for 2 min. The remaining steps of the RNA extraction was performed according to the protocol provided in the kit (Qiagen). Genomic DNA was eliminated by RNase-free DNase I treatment during the isolation procedure. After RNA extraction, the material was further treated by TURBO™ DNase, according to the protocol provided by the manufacturer (Ambion). At this point no trace of bacterial or host DNA could be detected in the qPCR analysis. The RNA concentration and quality were measured by NanoDrop (Thermo Scientific, Wilmington, DE, USA) and Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA), respectively. Quality requirements were: A260/A280 $\geq 1.8$ and RIN $\geq 5$. Samples not meeting this standard were discarded and new extractions performed. Most of the RIN scores were between 6 and 7, which indicated some degree of RNA degradation. However, as the integrity number was based on a mixture of pro- and eukaryotic RNA, with the latter comprising main part of the measured RNA, we assumed that this was primarily due to increased enzymatic processes in the infected eukaryotic tissue which would most likely not affect the integrity of bacterial RNA protected by the bacterial cell wall.

For each sample, 30 µg of total RNA was enriched for bacterial RNA applying the MicroEnrich Kit according to the supplied protocol (Ambion). Subsequently, one µg of the enriched RNA was amplified using a MessageAmp II-Bacteria kit (Ambion) according to the manufacturer’s instructions.

Preparation of labeled double-stranded DNA

Ten micrograms of total RNA from each sample was reverse transcribed using SuperScript II (Invitrogen, Carlsbad, CA) and Random Hexamer Primers (Invitrogen) according to the NimbleGen Arrays User’s Guide (Gene Expression Analysis v3.2). The generated cDNA was incubated with 1 µl of 4 mg/ml RNase A solution (Promega Corporation, Madison, WI) at 37°C for 10 min, and then phenol-chloroform extracted. Samples were centrifuged in Phase Lock Gel Tubes (5 Prime, Hamburg, Germany) at 12,000 x g for 5 minutes and precipitated with 80% ethanol. Pellets were air dried in a SpeedVac and rehydrated in 20 µl of ultrapure water (Ambion). Finally the samples were measured by NanoDrop to ensure that the cDNA met the following quality requirements: A260/A280 $\geq 1.8$ and A260/A230 $\geq 1.8$. NimbleGen One-Color DNA Labeling kit (NimbleGen Systems, Madison, WI) was used for Cy3 labeling of cDNA samples according to the NimbleGen Arrays User’s Guide. Briefly, 1 µg double-stranded cDNA was incubated for 10 min at 98°C with Cy3-random Nonamers and then quick-chilled in an ice-water bath for 10 min. The addition of 100 mM of deoxynucleoside triphosphates and 100 U of Klonef fragment (New England Biolabs, Ipswich, MA) was followed by incubation at 37°C for 2 h. The reaction was stopped by adding 0.1 volumes of 0.5 M EDTA, and the labeled cDNA was precipitated with isopropanol.

DNA microarrays

The arrays used in this project were based on the NimbleGen 12-plex platform, officially released in a news statement on Nov. 19, 2008. The custom probe set for the arrays was build around a set of 7 core genomes representing all publically available A. pleuropneumoniae and Actinobacillus succinogenes genomes in GenBank and RefSeq, which included draft genome sequences of A. pleuropneumoniae serotypes 2 and 6 [PMID: 18073190]. The array included 130,194 active probes excluding NimbleGen control probes. Each gene was covered by an average of 26.7 probes of an average size of 48 bp. The detailed construction of the array has been previously described [96] and the design is publicly available at NimbleGen (091013_DTU_Actino_sXRNA).

Hybridization and analysis of arrays

A hybridization kit (NimbleGen Systems) was used for the hybridization step. Cy3-labeled samples were resuspended in the recommended amount of hybridization buffer and denatured at 95°C for 5 min. Slides were placed in HX12 NimbleGen Mixer and 6 µl of sample loaded though the fill port. Hybridization was performed for 20 h at 42°C (NimbleGen Hybridization System 16). The arrays were washed using a wash buffer kit (NimbleGen Systems), dried in a microarray dryer (NimbleGen Systems), and scanned at a 5 µm resolution using the NimbleGens MS 200 scanner (NimbleGen Systems).

Quantitative real-time PCR (qPCR)

Gene quantification was performed with a Rotor-Gene 6000 (Corbett Research, Sydney, Australia). The primers were designed using Primer3 (v. 0.4.0) [97]. The sequences of the primers are listed in Table S6. Each PCR was performed in a 25 µl reaction mixture containing 12.5 µl QuantiTect SYBR Green PCR master mix (Qiagen, Hilden, Germany), a primer concentration of 0.3 µM and 7 ng of cDNA. Three biological replicates were included for each sample. The thermal cycling conditions were as follows: 15 min at 95°C, followed by 40 cycles of 30 s at 94°C, 20 s at 55°C, and 20 s at 72°C. Data collection was performed during each extension phase. Positive controls (DNA) and negative controls (distilled water) were included in each run. Control for DNA contamination was performed before linear amplification of the mRNA. Melting curve analysis was performed, which for all primer sets resulted in single product-specific melting curves.

In the qPCR analysis of amplified versus non-amplified A. pleuropneumoniae mRNA the target concentration for each amplicon was determined from an optimized standard curve (Table S7). The concentration 3600 pg/µl was used as inter-plate calibrator. The geometric means of the previously validated genes gldA and pykA were applied as reference genes for normalization [98].

For validation of the microarray data relative quantification was applied. The Excel-based relative expression software tool, REST 2009 (V2.0.13), was applied for group wise comparison and statistical analysis of the qPCR data (http://rest.genequantification.info/) [99]. The relative expression ratios were calculated by a mathematical model, which included an efficiency correction for real-time PCR efficiency of the individual transcripts [100]:

\[
\text{Ratio} = \left( \frac{E_{\text{target}}}{E_{\text{ref}}} \right)_{\text{target}} \times \left( \frac{E_{\text{ref}}}{E_{\text{target}}} \right)_{\text{ref}} \times \left( \frac{C_{\text{ref}}}{C_{\text{target}}} \right)
\]

The relative expression ratio of a target gene was computed based on its real-time PCR efficiencies ($E$) and the crossing point difference ($\Delta CP$) for an unknown sample versus a control (in this case we compared 6 hours p. i. versus 48 hours p.i.). For each gene, cDNA dilution curves were generated and used to calculate the individual real-time PCR efficiencies ($E = 10^{1/\text{slope}}$). The
geometric mean of two internal reference genes was used to correct the raw values for the genes of interest (Table S5). From the genes that displayed the least variations in expression between the 75 microarrays, two new references were chosen for normalization. These were the carbon storage regulator (cscD), belonging to the functional group “signal transduction mechanisms” and phosphomannomutase (manB) from the functional group “carbohydrate transport and metabolism.”

Microarray analysis

The data discussed in this publication have been deposited in NCBI’s Gene Expression Omnibus [101] and are accessible through GEO Series accession number GSE33999, (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE33999). Data analysis of the microarrays was performed in “RGUi” version 2.9.2 (2009-08-24) (http://cran.r-project.org/bin/windows/base/), using the package “Oligo”. The Robust Multiclip Average function was applied for normalization and index calculation of the microarray data [102]. By this method, the expression measure is given in log2 base. For each time point, the mean log2 expression values of the included pigs (three samples from each pig) are given in Table 1 and Table 2. A two-way analysis of variance (ANOVA) (serotype 2 versus serotype 6) and time (F2: variations between time points 6 h, 12 h, 24 h and 48 h p.i.) (Table S3).

Figure S1 shows a density plot of the 75 microarrays. Probe targets where no hybridization signal was detected (signal below 9 fold change) and clusters) [24].

Supporting Information

Figure S1 Density plot of the expression profiles from all 75 arrays used in this study. Each colored line reveals the distribution of signal for one specific array. The tall spike to the left clearly indicates genes either not present in the organism or not expressed at all. The softer hill-like spike to the right represents those genes experiencing at least a certain level of expression. Created using the density function in R, which uses Fourier transformations and Gaussian kernel estimates to derive the functions underlying the observed data. (PDF)

Table S1 Overview of pig samples included in the study. (PDF)

Table S2 Quantitative RT-PCR analysis of amplified versus non-amplified samples. (PDF)

Table S3 A. pleuropneumoniae genes displaying significant differential expression during the acute phase of infection. (PDF)

Table S4 The most highly and constitutively expressed genes of A. pleuropneumoniae during the first 48 h post experimental challenge. (PDF)

Table S5 Genes found to be differentially expressed in the present and other expression studies of A. pleuropneumoniae or H. influenzae. (PDF)

Table S6 List of primers used for quantitative real-time PCR. (PDF)

Table S7 R2 values and Efficiency of standard curves used in qPCR analyses. (PDF)

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Author Contributions

Conceived and designed the experiments: KK CF TKJ OA MB. Performed the experiments: KK TKJ. Analyzed the data: KK CF. Contributed reagents/materials/analysis tools: KK CF TKJ OA MB. Wrote the paper: KK CF TKJ OA MB.

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